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Unfolding of *in planta* activity of anti-*rep* ribozyme in presence of a RNA silencing suppressor

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A B S T R A C T

Antisense RNA ribozymes have intrinsic endonucleolytic activity to effect cleavage of the target RNA. However, this activity *in vivo* is often controlled by the dominance of antisense or other double-stranded RNA mechanism. In this work, we demonstrate the *in planta* activity of a hammerhead ribozyme designed to target *rep*-mRNA of a phytopathogen *Mungbean Yellow Mosaic India virus* (MYMIV) as an antiviral agent. We also found RNA-silencing is induced on introduction of catalytically active as well as inactive ribozymes. Using RNA-silencing suppressors (RSS), we demonstrate that the endonucleolytic activity of ribozymes is a true phenomenon, even while a mutated version may demonstrate a similar down-regulation of the target RNA. This helps to ease the confusion over the action mechanism of ribozymes *in vivo*.

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1. Introduction

The hammerhead ribozyme (hRz) is a class of catalytic RNA with characteristic hammerhead-like secondary structural motif. Though they were originally discovered in sub-viral plant pathogens, they have been recently shown to be associated with many other genomes as well [1,2]. They are among the well-studied class of ribozymes with known structure and catalytic chemistry [3–5]. The hRzs are relatively small in size with an ease of manipulation to design a sequence specific *trans*-cleaving motif against the RNA of interest containing "GUX" cleavage site [6]. These features have made them attractive tools for varied biotechnological applications including inhibition of viral genome replication. There are numerous reports of applications against various plant and animal viruses, including human immunodeficiency virus, hepatitis B virus, hepatitis C virus, dengue fever virus, influenza virus, SARS virus, herpes simplex virus, potyvirus, plum pox virus etc. [7–12]. Even though, other nucleic acid based anti-viral strategies, such as RNA-silencing, has gained impetus, the impact of ribozymes is

hard to ignore. In the battle between the hosts and viruses, the latter species have evolved to encode for proteins or nucleic acids with the potential to suppress RNA-silencing [13–15]. But in case of ribozyme attack, no such counter-attack strategies from viruses are known, thereby, attracting researchers to design ribozyme based antiviral strategies [10–12,16–18].

The use of ribozymes as antiviral agents has been limited to RNA viruses with only a little exploration of its effect on DNA viruses. Infact, DNA viruses offer a greater opportunity for 'Rz's to target the sub viral RNA species encoding the essential proteins instead of the whole viral genome [10,19,20]. In our previous report, we have designed a hammerhead ribozyme against Rep protein encoding RNA of *Mungbean Yellow Mosaic India virus* (MYMIV), a member of family geminiviridae [20]. The family *Geminiviridae* is one of the largest and most important families of phytoviruses. They have single-stranded circular DNA genomes encoding genes that diverge in both directions from a virion strand origin of DNA replication. The Geminiviral encoded proteins have the potential to redirect host machineries and processes to establish a productive infection. These interactions reprogramme plant cell cycle and transcriptional controls, inhibit cell death pathways, interfere with cell signaling and protein turnover and suppress defense pathways [21,22]. As a consequence, these group of viruses cause huge agro-economical losses worldwide and hence special attention for the development of antiviral strategy against them

needs to be deployed .The ribozyme technology, as an antivirus strategy, could be of major significance for the geminiviridae for the following reasons. First, like most RNA viruses and some viroids, DNA containing geminiviruses might induce eventual virus resistance via RNA-silencing mechanism, and the additional introduction of ribozyme could complement the host defense [21–23]. Secondly, the geminiviruses, including MYMIV, encode for RSSs that inhibit one or more distinct steps of RNA-silencing pathways [24–26]. Thus ribozyme strategy might serve as second line of defense in cases where the RNA-silencing strategy alone might fail to restrict the virus.

In this work, we have demonstrated *in planta* activity of the anti-*rep* ribozyme (Rz) as an antiviral strategy to MYMIV. We have also addressed the issue whether the resultant down-regulation of the target gene is mediated by the catalytic activity using the catalytically mutant anti-*rep* ribozyme (mRz). The mutation hampered the Mg^{2+} binding of Rz [20]. In this particular event, the ribozyme activity was over-shadowed by the host RNA-silencing activity. To unfold the true ribozyme activity, we used the RSS that suppressed the endogenous RNA-silencing effect. To our best knowledge, this is the first report that uses RSS to visualize ribozyme activity. The designed anti-rep ribozyme could successfully demonstrate antiviral activity in the presence of RSS.

2. Materials and methods

2.1. Plant growth

Tobacco plants (*Nicotiana tabacum* cv. Xanthi) were grown in greenhouse under controlled condition of 25 °C and 16 h daylight.

2.2. Vector construction and transformation

The chemically synthesized oligonucleotides coding for ribozyme and mutant ribozyme [20], were annealed and directly cloned down-stream of CaMV-35S promoter in pRT100 and subsequently mobilized into a binary vector pCAMBIA1391Z at *HindIII* restriction site, as an expression cassette. The plasmids were designated as Rz, mRz and empty vector (EV) for pCAMBIA1391Z ribozyme, mutant ribozyme and vector alone, respectively. Target gene and viral amplicon was provided through Cam/VA$_{AC2M}$/GFP vector [25,27], a MYMIV (NCBI accession No. AF126406) based mini-viral amplicon with a non-sense mutation at AC2 gene and tagged with a reporter gene mGFP5 cloned in pCAMBIA1391Z vector [detailed vector construction was described in ICGEB activity report (2004), PhD thesis of M. N. Islam (2005)]. RSSs were provided asMYMIV-AC2 orFHV-B2 ORFs cloned in pBI121 binary vector between 35S-promoter and *Nos*-terminator using *BamHI-SacI* and *XbaI-BamHI* restriction sites, respectively. All the constructed vectors were transformed into *Agrobacterium tumifaciens* LBA4404 for agro-infiltrations.

2.3. Agrobacterium culture and co-infiltration

Agrobacterium constructs were grown in YEM broth under kanamycin antibiotic selection (50 mg/ml) at 30 °C 250 rpm till the OD reaches 0.8–1.0 at λ_{max} 600. Cells were collected by centrifugation at 3000 rpm for 5 min followed by their resuspension in fresh YEM broth without antibiotic. The homogenous cell suspension was infiltrated into the selected young leaves [25,28] (using a 5 ml needleless syringes). The amount of cell suspension used for double-co-infiltration with Cam/VA$_{AC2M}$/GFP and Rz (or mRz or EV) was mixed in a ratio of 1:2 and 1:2:1 for triple-co-infiltrations with Cam/VA$_{AC2M}$/GFP, Rz and RSS. All the agro-infiltrations were carried out in wildtype tobacco cv. Xanthi leaves of 2 months old

plant. The infiltrated leaves were plucked from the plant 12 days post infiltration (dpi).

2.4. RT-PCR and PCR analysis

Leaf samples were collected at 12 dpi total RNA was extracted following trizol-RNA isolation protocol (Invitrogen) and semi-quantitative RT-PCR analysis were carried out to monitor the levels of MYMIV-rep mRNA [25]. Oligonucleotides used for the semi-quantitative RT-PCR were as follow:

Rep$_{fwd}$: 5′-TACGGTTCCCTTCCAGCAAAACGA-3′
Rep$_{rev}$: 5′-TCAATTCGAGATCGTCGAATTGCT-3′

Genomic DNA was isolated from the 12 dpi leaf samples by standard CTAB method and treated with DpnI restriction enzyme for 6 h at 37 °C [25,29]. PCR methodology was used to semi-quantify the amount of viral replicon in each infiltrated samples [25,29]. Oligonucleotides used for the semi-quantitative PCR were as follows:

GFP$_{fwd}$: 5′-GCTCTAGACCATGGCAAGTAAAGGAGAAG-3′
Rep$_{rev}$: 5′-TCAATTCGAGATCGTCGAATTGCT-3′

The RT-PCR and PCR were carried out for 22 cycles with actin gene as the internal control. The amplification products were separated on 1% agarose gels and the band intensities were quantified using Quantity One (BioRad, USA) software.

2.5. Small RNA analyses by Northern blot

Small RNAs extracted from 12 dpi leaf samples were separated on 7 M Urea-15% PAGE [25] and transferred to Hybond N$^+$ membrane (GE Healthcare, USA) by electroblotting in $1\times$ TBE buffer at 0.32 mA cm^{-2} for 50 min [28]. The Northern blot was probed with P$^{32}\alpha$-labeled dCTP MYMIV-*rep* prepared by nick translation of *rep* template using Invitrogen kit. The bands emerged post hybridization were estimated for relative intensities by densitometric scanning using Typhoon 9210 scanner and analyzed by Image Quant TL software (GE Healthcare, USA).

2.6. In vitro transcription and cleavage of rep transcript by ribozyme

The chemically synthesized oligonucleotides coding for Rz and mRz were annealed and ligated to the double digested (SmaI and BamHI) pSGI vector down-stream of T7 promoter, designates as pSGI-Rz and pSGI-mRz [20]. Similarly, rep gene was cloned in between the BamHI and HindIII sites of pET28a, denoted as pET28a-rep. The constructs were linearized (pSGI-Rz/ pSGI-mRzwith restriction enzyme *BamHI* and pET28a-repwith *ClaI*) to serve as templates for the *in vitro* transcription. Riboprobe *in vitro* transcription kit (Promega) was used to *in vitro* transcribe unlabeled Rz (or mRz) and α^{32}P-UTP (3000 Ci/nmol) radioactively labeled *rep* transcript [20]. The *rep* and Rz (or mRz) were mixed and cleavage reaction was carried out under standard cleavage conditions and analyzed on 6% denaturing PAGE to separate the target from the products [20]. The gel was vacuum dried, exposed to X-ray film (BIOMAX MS, Kodak, USA) and the autoradiographic bands were quantified by Alpha Imager software (Bio-Rad).

3. Results and discussion

The hammerhead ribozyme (Rz) directed against the *rep*-mRNA of MYMIV with *in vitro* endonucleolytic cleavage activity under physiological Mg^{2+}concentration and pH had been reported earlier from our group [20]. The inhibitory effect of Rz on geminiviral DNA replication was also observed in surrogate host *Saccharomyces cerevisiae* [20]. This encouraged us to investigate the Rz activity

in planta, an essential determining step in translating this technology for field applicability.

In this report, *in planta* efficiency of the anti-*rep*-ribozyme was investigated with agro-infiltration based transient bioassay in wildype tobacco cv. Xanthi [25,30]. We have used a specially designed MYMIV based vector Cam/VA$_{AC2M}$/GFP that mimics viral replication and expresses rep-RNA under its natural promoter [25,29]. In addition, the tagged GFP reporter gene helps in easy visualization of the Rz effect on the target *rep*-RNA and viral amplicon (the activity of the Rz will lead to a decrease in green fluorescence of GFP). The Cam/VA$_{AC2M}$/GFP was co-infiltrated with Rz or the empty vector (EV) in *Nicotiana xanthi* leaves and analyzed on 12 dpi. Under UV illumination, the control empty vector with Cam/VA$_{AC2M}$/GFP infiltrated patches showed green fluorescence of GFP while the ribozyme encoding vector withCam/VA$_{AC2M}$/GFP did not show GFP fluorescence but showed only red colour [due to chlorophyll auto-fluorescence (Fig. 1A)], suggesting the possible *in planta* Rz activity. Since, Rz was designed for endonucleolytic activity against *rep*-RNA, we measured the *rep*-RNA level. The *rep*-RNA specific semi-quantitative RT-PCR analysis showed around 40% reduction in the rep-RNA level (Fig. 1B: Lanes 1 and 3) compared to the EV co-infiltration. As the Rep protein is the most essential component of MYMIV replication machinery, the reduction in the level of *rep*-RNA should adversely affect the viral amplicon accumulation level. Semi-quantitative PCR based strategy was employed to determine the viral amplicon level and ~40% reduction in the accumulation of viral amplicon was observed in Rz treated samples in comparison to the EV control (Fig. 1C: Lanes 1 and 3). Thus, the results indicated that the Rz down-regulated the target RNA *rep* and consequent the viral amplicon accumulation in planta. However, in order to ascertain that the observed reductions were due to the catalytic (endonucleolytic) activity of ribozyme, the above-mentioned mini-viral vector was also co-infiltrated with the catalytically inactive ribozyme (mRz).

Ribozyme catalysis was disabled by altering the sequence at two base pairs (T19 → C; C37 → T) in the catalytic core [20]. As desired, mRz did not show *in vitro* cleavage activity even after prolonged exposure (Fig. 2A). The mRz also caused a reduction (~40%) in *rep*-RNA and viral amplicon (Fig. 1) *in planta*. Therefore, Rz and mRz activities against the target RNA and viral amplicon were indistinguishable (Fig. 1). The catalytic activity appeared redundant, suggesting that the observed in planta Rz activity was possibly not a true ribozyme function. This behavior was completely unexpected and ran contrary to our speculations. However, it raised an important question to be addressed: What could be the underlying mechanism behind the observed reduction mediated by Rz and mRz? Therefore, we investigated possible involvement of other RNA based anti-gene strategies in this process. The three major forms of RNA based anti-gene mechanisms considered here are those of ribozyme, RNA silencing and anti-sense constructs.

RNA-silencing is induced by transient dsRNA formation and subsequent down-regulation of the target-RNA is caused by target-RNA specific small interfering (si) RNA in presence of
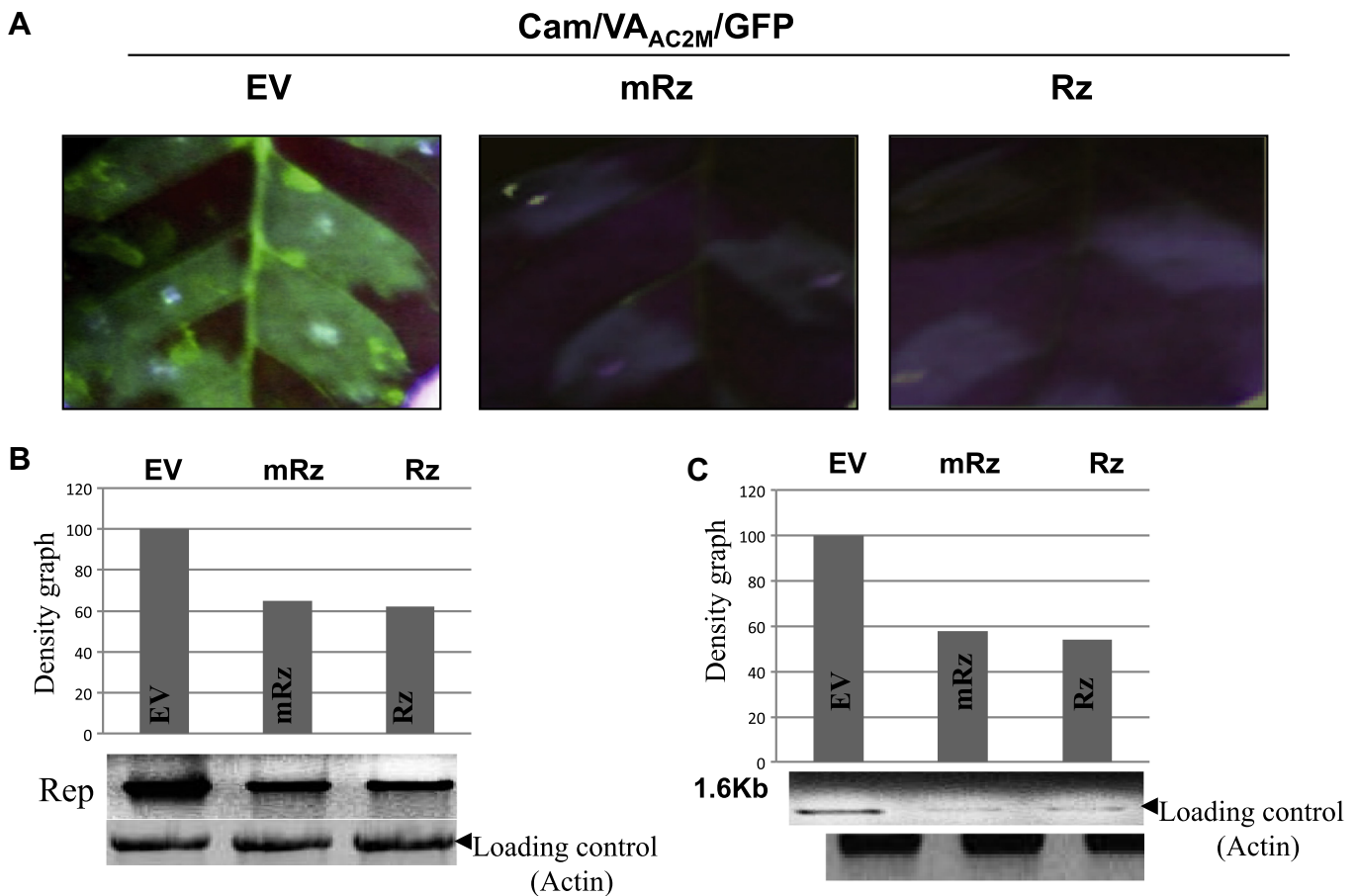

Fig. 1. (A–C) Evaluation of *in planta* efficiency of ribozyme through agro-infiltration. (A) Leaf pictures of wildtype tobacco cv. Xanthi leaves co-infiltrated with Cam/VA$_{AC2M}$/GFP and Rz or mRz or empty vector (EV) over UV trans-illuminator. Each of the leaves was labeled at the top for the infiltrated construct. (B) The RT-PCR amplification from the c-DNA template prepared from the co-infiltrated samples with actin amplification as the loading control. (C) The semi-quantitative PCR represents 21 cycles amplification of 1.6 Kb with their respective actin amplification as loading control. The density graphs in (B) and (C) was plotted as relative value to EV infiltration (100%) after normalizing with the loading control.

complex host machinery. The Mfold based bio-informatics tool [31] predicted a stable dsRNA (thermodynamic value, $\Delta G = -48.73$ kcal/mol) secondary structure from the hybridization of rep-RNA with Rz or mRz (Fig. 2B). In ribozyme the internal guide sequence (IGS) recognizes and hybridizes with the target RNA to generate transient dsRNA and the mutation in mRz was placed only in the catalytic core (not a part of the hybridization structure). Hence, similar secondary structure was obtained for rep-Rz and rep-mRz (Fig. 2B). The dsRNA generated in this way could possibly induce the host RNA-silencing mechanism. Interestingly, we observed the 22 nt length small RNA against rep-RNA in the infiltrated 12 dpi leaf samples in case of both Rz and mRz (Fig. 2C: Lanes 1 and 2). The nature of siRNA was also confirmed by using the MYMIV-AC2, which is a known RNA-silencing suppressor and inhibits the biogenesis of siRNA [32]. A remarkable decrease in small RNA formation was observed (Fig. 2C: Lanes 3 and 4), upon agro-triple-infiltration of Cam/VA$_{AC2M}$/GFP + Rz (or mRz) + MYMIV-AC2. It was interesting to observe higher amount of siRNA formation with mRz, the reason for this was not clear at the moment. However, the durability of dsRNA formation with mRz could be speculated and might cause this higher yield of siRNA. Thus, RNA-silencing seems to be the probable mechanism underlying the observed in planta activity of Rz and mRz. Therefore, it would be interesting to determine the Rz and mRz activity in a condition where RNA-silencing mechanism is deficient or suppressed.

Yeast species, *Saccharomyces cerevisiae*, exhibits all RNA based anti-gene principles except for RNA silencing. Interestingly, in host system *S. cerevisiae*, we observed adverse effect against the rep-RNA and viral amplicon, only with Rz [20]. Next, we created a RNA-silencing suppressed system within the experimental plant tobacco cv Xanthi by co-infiltrating a RNA silencing suppressor, MYMIV-AC2 [27]. In the absence of RNA-silencing mechanism as well, Rz retained its activity and led to loss of GFP fluorescence, along with reduction in rep-RNA levels and viral accumulation by almost 40% (Fig. 3A and B). However, mRz showed complete loss of antigene activity in presence of MYMIV-AC2 and was equivalent to the empty vector control (Fig. 3A and B). The above experiment was also repeated in presence of another RSS of heterologous origin, *viz.*, the insect virus *Flock House Virus* (FHV) encoded B2 protein [14]. In presence of FHV-B2 as well, Rz showed inhibitory activity against the target rep-mRNA and accumulation of the viral amplicon while the mRz behaved like an empty vector control. Therefore, the Rz mediated activity in this event was not due to any RNA-silencing (Fig. 3) and could be primarily ascribed to its endonucleolytic activity. Furthermore, the above observations clearly indicated that the principal mechanism for the mRz activity was only RNA-silencing.

Ribozyme technology despite various successful application is often challenged as an allied RNA based anti-gene strategies [33]. Intriguingly, our initial results also led us to regard Rz activity as a function of RNA-silencing (Figs. 1, 2B and C). However, the true in planta ribozyme activity was unfolded upon application of RSS. Thus, the RNA- silencing suppressor, which is essentially a pathogenicity factor for the virus to counter-attack the host and establish disease, could be used as a novel biotechnological tool to uncover another biological mechanism. In addition, a survey of past work in this field suggested to us that down-regulation caused by catalytically mutated ribozyme is usually attributed to the 'antisense effect' [34]. Interestingly, mRz designed by us showed no antisense effect (as no mRz activity was observed in *S. cerevisiae*, an antisense proficient system [20]) but induced RNA-silencing to down-regulate the targeted RNA.

Thus, the tested Rz showed in planta inhibitory potential against the DNA virus MYMIV and might be developed as an antiviral strategy. Although, in the recent past, RNA-silencing had been
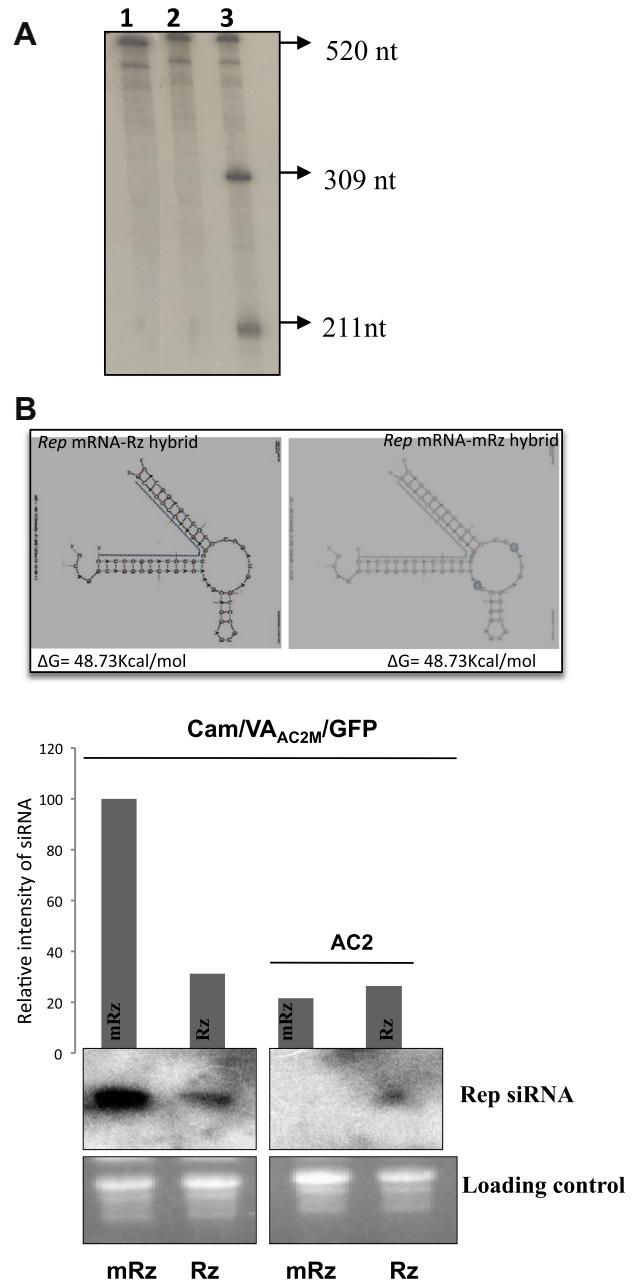

Fig. 2. *In vitro* cleavage reaction, structure prediction and siRNA analysis. (A) Autoradiogram showing comparative cleavage potential against in vitro transcribed 10 fmol radiolabelled rep-RNA transcript (Lane 1) by 5 fmol mRz (Lane 2) and 5 fmol Rz (Lane 3) at pH 7.5 and 1 mM Mg^{2+} concentration. Arrows show the intact and the cleaved rep transcripts, where substrate (520 nt rep transcript), product-1 (309 nt) and product-2 (211 nt). (B) Mfold predicted secondary structure of Rz and mRz hybridized to rep-mRNA along with their respective ΔG-values. (C) Evaluation of siRNA level corresponding to the rep-mRNA from the sample co-infiltrated with Cam/VA$_{AC2M}$/GFP/ + Rz or mRz alone (Lanes 1 and 2) and in presence of the MYMIV-AC2 (Lanes 3 and 4) by Northern blot analysis. The density graph was plotted after normalizing the band intensity of siRNA northern blot band (top panel) with their respective EtBr-stained 5SRNA + tRNA bands as loading control and presented as relative value considering the mRz infiltrated sample value as 100%.

developed as a very important and useful technology, the advantage of ribozyme technology cannot be disregarded. Considering most of the viruses have been found to encode RSS as a pathogenicity factor, the ribozyme technology becomes further important. In case of HIV, a report on comparison of siRNA and ribozyme targeting the same region of HIV-1 pol gene appeared and the latter was found to be more effective against the viral accumulation [35].
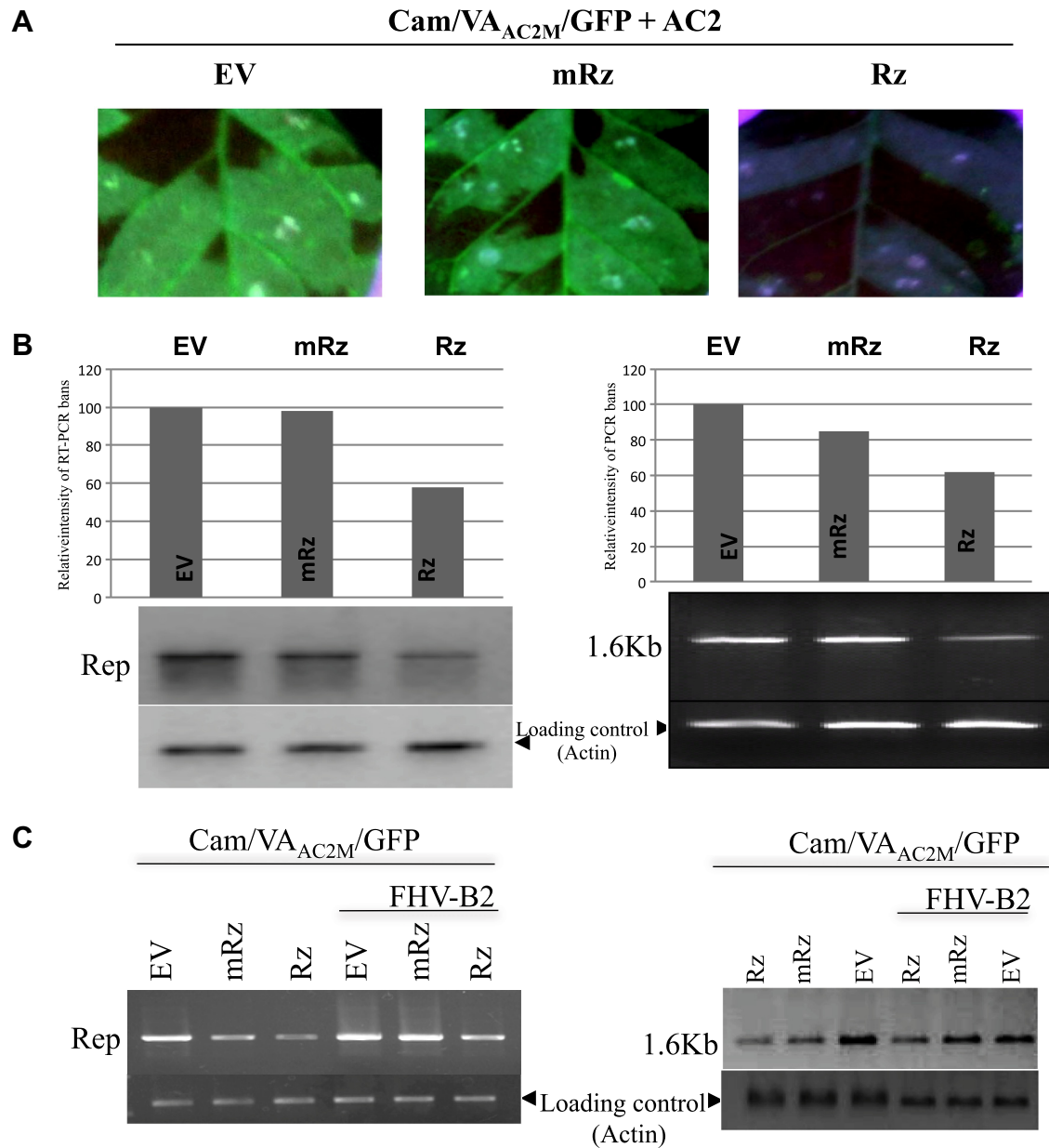
Fig. 3. (A–C) Evaluation of *in planta* Rz and mRz results in presence of RSS. (A) Leaf pictures of wildtype tobacco cv. Xanthi leaves co-infiltrated with Cam/VA$_{AC2M}$/GFP + EV or Rz or mRz along with MYMIV-AC2, over UV trans-illuminator. Each of the leaves was labeled at the top for the infiltrated constructs. (B) Right panel represents the RT-PCR amplification with rep-primers from the c-DNA template prepared from the co-infiltrated samples, with actin amplification as the loading control. Left panel represents the semi-quantitative PCR of 21 cycles amplification of 1.6 Kb band with respective actin amplification as loading control. The density graph was plotted as relative value to EV infiltration (100%) after normalizing with the loading control for both the right and left panels. (C) Viral amplicon titer determination by RT-PCR (right panel) and PCR (left panel) form the co-infiltrated samples of Cam/VA$_{AC2M}$/GFP + EV or Rz or mRz alone or with FHV-B2. Respective loading control of actin amplification has been presented in the bottom panel.

Thus, ribozyme technology may be valuable for its application as antiviral strategy alone or in combinatorial approaches to compliment and/or supplement the integrative virus management strategies.

Conflict of interest statement

None declared.

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